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Enumeration and Characterization of *Aeromonas hydrophila* and *Aeromonas caviae* Isolated from Grocery Store Produce

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Starch-ampicillin agar was used to quantitatively isolate *Aeromonas* sp. from retail grocery store produce. All produce sampled, including parsley, spinach, celery, alfalfa sprouts, broccoli, and lettuce, contained *Aeromonas* sp. In most instances, the count of *Aeromonas* sp. increased 10- to 1,000-fold during 2 weeks of storage at 5°C. Eleven (92%) of 12 kinds of produce yielded cytotoxic *Aeromonas* sp. Identification as *Aeromonas hydrophila* was the strongest indicator of cytotoxicity, and all 29 (100%) *A. hydrophila* isolates and 1 (6%) of 16 *A. caviae* isolates were cytotoxic. Twenty-seven (90%) of 30 cytotoxic *Aeromonas* sp. strains produced hemolysins. Strong correlations were also noted between ability to produce cytotoxin and positive Voges-Proskauer, lysine decarboxylase, and sorbitol fermentation reactions. It appears that grocery store produce is a potentially significant source of cytotoxic *Aeromonas* sp. and should be considered in the epidemiology of *A. hydrophila* gastroenteritis.

More than a decade ago, *Aeromonas* species were implicated in human gastroenteritis ranging from mild diarrhea to life-threatening choleralike illness (2, 11). Since those reports, multiple investigators have reported that this species appears to be a significant enteric pathogen (1, 5, 10, 17). However, controversy still exists concerning the role of *Aeromonas* sp. in acute gastroenteritis (2, 14, 16).

A variety of extracellular virulence factors produced by *Aeromonas hydrophila* support the epidemiological associations indicating that this is a bona fide enteric pathogen. Using ligated rabbit ileal loops (28), suckling mice (7), perfusion of rat jejunum in vivo (30), or cell cultures (13), investigators have demonstrated the production of a cytotoxigenic enterotoxin. In addition, Ljungh and Wadstrom (22) suggest that hemolysin, protease, and endotoxin production may influence virulence, and Jiwa (20) suggests that cell surface hydrophobicity may be a determinant of intestinal attachment.

Most studies involving the ecology of *A. hydrophila* gastroenteritis have concentrated on its transmission in contaminated water supplies (27, 29). However, Buchanan and Palumbo (4) implicated *Aeromonas* sp. as potential food-poisoning agents. *A. hydrophila* is psychrotrophic and has been associated with the spoilage of refrigerated (5°C) animal products including chicken, beef, pork, lamb, fish, oysters, crab, and milk (15, 18, 21, 23, 31).

Due to the absence of a suitable isolation medium, data concerning quantitation of *Aeromonas* sp. in foods are generally lacking. Recently, Palumbo et al. (23) developed a simple, direct plating medium that enables the isolation and quantitation of *Aeromonas* sp. from foods obtained from the retail market.

The purpose of our study was to use starch-ampicillin agar to investigate grocery store produce for *Aeromonas* sp. In addition, we characterized these *Aeromonas* isolates by temperature tolerance, biochemical characteristics, hemolysin production, and cytotoxic enterotoxin production.

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MATERIALS AND METHODS

Foods. Leafy produce samples were purchased at local retail markets and immediately transferred to the laboratory, where they were sampled on the day of purchase and after 7 and 14 days of storage at 5°C. Sampling was achieved by aseptically transferring 10 g of the produce to a sterile 500-ml glass blender container (Ederbach 8470), adding 90 ml of sterile 0.01 M phosphate-buffered saline (pH 7.2), and blending in a heavy-duty blender (Waring) at high speed for 2 min. Dilutions were then made with 0.01 M phosphate-buffered saline (pH 7.2) and surface (0.1 ml) or pour (0.1 ml) plated, using the appropriate test media.

Media. Total aerobic plate counts were determined with appropriate dilutions (30 to 300 CFU) in plate count agar (Difco Laboratories) pour plates. Plates were incubated at room temperature for 1 week.

The starch-ampillicin agar was prepared according to Palumbo et al. (23). Dilutions of food samples were surface plated and incubated at 28°C for 24 h. After incubation, the plates were flooded with approximately 5 ml of Lugol iodine solution (12), and amylase-positive colonies were scored as presumptive *Aeromonas* sp.

Biochemical identification to species. After quantitation, three presumptive *Aeromonas* isolates were identified biochemically, using oxidase, catalase, DNase, no growth in 1 and 6.5% NaCl and in lysine decarboxylase (LDC) broth (GIBCO Diagnostics), and API 20E analysis (Analytab Products). Additional tests included esculin hydrolysis, growth in KCN broth, Voges-Proskauer (VP) reaction, and gas from glucose, as recommended by Popoff (25).

Temperature tolerance. The effect of various incubation temperatures (42, 35, 20, 12, and 5°C) on the growth/survival of the *Aeromonas* strains was determined by optical density measurements according to the technique described by Palumbo et al. (24). Single brain heart infusion broth (3.0 ml) tube cultures in Spectronic 20 tubes (12 by 100 mm), inoculated with ca. 2×10^3 CFU of each isolate per ml, were used in conjunction with incubation without agitation. Every 24 h, growth was followed by optical density measurements

TABLE 1. *Aeromonas* sp. and total aerobic count of grocery store produce at time of purchase and after 7 and 14 days at 5°C

Produce	Toal count, mean (CFU/g)[a]					
	Time of purchase		Day 7		Day 14	
	Aerobes	*Aeromonas* sp.	Aerobes	*Aeromonas* sp.	Aerobes	*Aeromonas* sp.
Red-leaf lettuce						
Sample 1	1.7×10^6	7.8×10^2	1.4×10^8	1.1×10^3	5.6×10^7	4.5×10^3
Sample 2	3.7×10^6	1.6×10^3	1.6×10^8	7.5×10^2	3.5×10^7	4.6×10^3
Green-leaf lettuce						
Sample 1	5.6×10^4	1.0×10^2	4.7×10^6	ND[b]	1.6×10^7	8.0×10^3
Sample 2	1.3×10^6	7.3×10^3	6.0×10^6	ND	2.1×10^7	1.1×10^4
Parsley						
Sample 1	1.1×10^4	ND	3.7×10^7	3.2×10^4	1.7×10^7	3.5×10^5
Sample 2	1.5×10^7	ND	7.0×10^6	7.6×10^3	8.7×10^7	6.2×10^5
Spinach						
Sample 1	7.2×10^6	ND	1.2×10^8	ND	7.9×10^6	4.5×10^3
Sample 2	9.1×10^6	5.1×10^3	8.8×10^6	1.2×10^3	5.8×10^7	3.7×10^4
Endive						
Sample 1	4.6×10^6	ND	2.7×10^6	ND	8.6×10^7	ND
Sample 2	2.5×10^7	ND	6.6×10^6	ND	3.5×10^7	1.5×10^2
Celery						
Sample 1	5.2×10^5	3.6×10^3	3.3×10^6	ND	3.4×10^8	7.9×10^3
Sample 2	1.9×10^6	ND	1.1×10^8	5.5×10^2	3.7×10^7	4.3×10^3
Escarole						
Sample 1	3.5×10^4	ND	2.9×10^7	9.9×10^3	8.7×10^8	4.5×10^4
Sample 2	1.8×10^7	ND	1.0×10^7	ND	2.1×10^7	1.1×10^3
Romaine						
Sample 1	3.5×10^5	ND	3.1×10^7	2.2×10^3	4.0×10^7	2.5×10^3
Sample 2	2.5×10^7	1.0×10^2	7.1×10^6	1.0×10^2	3.2×10^6	1.0×10^2
Alfalfa sprouts						
Sample 1	8.0×10^7	ND	2.3×10^8	6.6×10^3	3.3×10^8	5.6×10^4
Sample 2	3.0×10^8	2.3×10^4	4.2×10^8	2.4×10^3	1.0×10^9	3.1×10^5
Boston lettuce						
Sample 1	2.8×10^6	ND	2.1×10^6	ND	8.2×10^7	1.1×10^5
Kale						
Sample 1	3.6×10^6	ND	2.7×10^6	2.0×10^2	9.7×10^7	2.8×10^5
Broccoli						
Sample 2	3.2×10^4	ND	2.2×10^8	5.5×10^2	3.4×10^8	5.7×10^5

[a] $n = 2$.
[b] ND, Not detected ($<10^2$ per g).

read at 600 nm in a Spectronic 20 spectrophotometer (Coleman) against an uninoculated tube of the same medium. Growth was considered positive if the optical density of the culture increased 0.1 U.

Preparation of cell-free supernatants. *Aeromonas* strains were propagated in 8 ml of brain heart infusion broth incubated at 35°C with agitation at 100 rpm for 18 to 24 h (13). The broth cultures were cleared by centrifugation at 10,000 × *g* for 30 min at 4°C, followed by filtration through a 0.45-μm filter (Millipore Corp.). Cell-free supernatants were stored at 4°C for use within 1 day (7).

Cytotoxin assay. Tests for cytotoxin were performed with culture supernatants added to separate HeLa and HEp-2 cell monolayers maintained in minimal essential medium containing 5% fetal calf serum. The supernatants were added at a final dilution of 1:5 to monolayers in 96-well microtiter plates (Nunclon) as previously described (13). Following an 18- to 24-h incubation at 35°C under 5% CO_2 and 90% relative humidity, the cells were examined for cell death. Only supernatants which caused 100% detachment were considered cytotoxin positive. Vital dye exclusion was not needed to confirm cell death because of the easily recognizable morphological cell changes, including cell shrinkage, loss of adherence, and pyknotic nuclei. Brain heart infusion broth diluted with minimal essential medium served as a negative control (13).

Hemolysin assay. Hemolysin activity was detected with 100-μl volumes of doubling dilutions of cell-free broth in 0.01 M phosphate-buffered saline (pH 7.2). These were added to equal volumes of a 1% suspension of rabbit erythrocytes in microtiter trays (Linbro) and incubated at 37°C for 1 h, followed by 1 h at 4°C (7). Filtrates causing lysis in >50% of the erythrocytes on visual examination were scored as hemolytic. Hemolysis was recorded as the reciprocal of the dilution of test material needed to produce 50% hemolysis. Values of >2 were considered positive.

RESULTS

Grocery store produce survey. The sampling protocol had a lower limit of detection of 100 *Aeromonas* sp. per gram (23). The *Aeromonas* sp. and total aerobic counts observed are summarized in Table 1.

Aeromonas sp. were detected in all grocery store produce samples except endive sample 1. The numbers of *Aeromonas* sp. recovered at the time of purchase ranged from 1.0×10^2 to 2.3×10^4 per g. After refrigeration for 14 days, *Aeromonas* recovery generally increased 10- to 1,000-fold. In a number of samples, concentrations of *Aeromonas* sp. increased to $>10^5$ per g by day 14.

Three representative amylase-positive colonies were isolated from the starch-ampicillin plates from each sample, and biochemically distinct organisms were identified to species. Twenty-nine (48%) of 61 total isolates were identified as *A.*

TABLE 2. Relationship between biochemical characteristics and cytotoxin production by 45 *Aeromonas* sp. isolated from grocery store produce

Character	Total isolates positive in assay [no. (%)]	Cytotoxin (n = 30) [no. (%)]	No cytotoxin (n = 15) [no. (%)]
VP reaction	29 (64)	29 (97)[a]	0
LDC[b]	28 (63)	28 (93)[a]	0
Sorbitol	21 (47)	21 (70)[a]	0
Rhamnose	1 (2)	1 (3)	0
Amygdalin	17 (38)	8 (27)	9 (60)
Arabinose	42 (93)	27 (90)	15 (100)

[a] $P < 0.001$, Fisher's exact probability test.
[b] LDC reaction from broth (GIBCO) after 24 h at 37°C.

hydrophila, 16 (26%) were identified as *A. caviae*, and 16 (26%) could not be assigned to any of the three motile and mesophilic *Aeromonas* species.

Temperature tolerance. The *A. hydrophila* and *A. caviae* isolates were able to grow over a temperature range of 35 to 5°C. At 42°C, only one (2%) of the isolates, an *A. caviae*, grew by day 14. All *Aeromonas* strains showed growth in 1 day at 35 and 22°C. At 12°C, growth slowed, but all strains grew within 48 h. Growth at 5°C was considerably slower, but again all strains of *A. hydrophila* grew in 9 to 11 days, and all strains of *A. caviae* grew in 10 to 13 days.

Cytotoxin and hemolysin production. HeLa cell lines have been routinely used for prior toxin studies but are more expensive than the HEp-2 cell lines (13). For this reason, we included HEp-2 cell lines and found that results correlated 100% of the time. Twenty-nine (100%) *A. hydrophila* isolates, but only 1 (6%) of 16 *A. caviae* isolates, were positive for cytotoxin.

In one instance, the undiluted supernatants of the endive *A. caviae* isolates produced rounding and loss of adherence of the HeLa cells. These cell changes were morphologically distinct and did not resemble those caused by cytotoxin. Cumberbatch et al. (13) believed this effect may be due to a cytotonic enterotoxin similar to *Escherichia coli* or cholera enterotoxins. However, this was not investigated.

Hemolysin production was positive in 26 (90%) of 29 broth filtrates of cytotoxic *A. hydrophila*. One (6%) of 16 *A. caviae* broths produced hemolysin, and this isolate also produced cytotoxin. Filtrates producing rounding or no effect in HeLa cells did not cause rabbit erythrocyte hemolysis.

Cytotoxic *A. hydrophila* isolates were recovered from 10 (83%) of 12 kinds of produce sampled, while cytotoxic *A. caviae* isolates were recovered only from alfalfa sprouts. Collectively, cytotoxic and hemolytic *Aeromonas* sp. were recovered from 11 (92%) of 12 kinds of produce.

Classification by hemolysin assay. There were 30 cytotoxic and 15 noncytotoxic strains of *Aeromonas* sp. isolated from grocery store produce. Of the 30 cytotoxic strains, 27 (90%) produced hemolysin, while all noncytotoxic strains were negative. This difference was highly significant by Fisher's exact probability test ($P < 0.001$).

Classification by biotype. All 45 *Aeromonas* isolates were tested in the API 20E system. Of the variable characteristics, only VP reaction and sorbitol fermentation correlated well with cytotoxin production (Table 2). A strong correlation between positive LDC and cytotoxin production occurred when cultures were tested in broth tube cultures (GIBCO).

The VP reaction was positive for 29 (97%) of 30 cytotoxic *Aeromonas* isolates, while none of the cytotoxin-negative isolates was positive. The only VP-negative cytotoxic isolate was *A. caviae* from alfalfa sprouts. Twenty-eight (93%) of 30 cytotoxic isolates were lactate dehydrogenase positive versus none of 15 noncytotoxic isolates. Likewise, 21 (70%) of 30 cytotoxic isolates fermented sorbitol, while no cytotoxin-negative isolates did. By Fisher's exact probability test, these differences were highly significant ($P < 0.001$).

DISCUSSION

The starch-ampicillin agar (23) used to quantify *Aeromonas* sp. was originally developed for use with products of animal origin; however, this medium performed well with grocery store produce. False-negative organisms were not encountered. *Vibrio* sp., which were eliminated by growth in 1% NaCl, were the only false positives.

The presence of significant populations of *Aeromonas* sp. in virtually all produce samples quantitatively establishes this organism as ubiquitous in grocery store produce. Increases in the numbers of *Aeromonas* sp. after 14 days of incubation at 5°C confirm the findings of Palumbo et al. (23, 24) that *Aeromonas* sp. are psychrotrophic organisms.

The growth of *A. hydrophila* and *A. caviae* in refrigerated (5°C) grocery store produce and meat samples (23) implicates *Aeromonas* sp. as potential food pathogens, and grocery store produce may represent an important vehicle of transmission. The numbers of recovered *Aeromonas* sp. represented only small portions of the total bacterial flora of the produce. It is unknown whether these inoculum sizes would be sufficient to cause disease.

In our study, 100% of environmental strains grew at 5°C, while in contradistinction, only 71% of clinical isolates grew at that temperature in a study by Palumbo et al. (24). Also, 100% of environmental isolates grew within 48 h at 12°C, while clinical isolates in Palumbo's study took 72 to 96 h for growth. Palumbo et al. (24) had 14 (82%) of 17 clinical isolates grow at 42°C, whereas only 1 (2%) of 45 environmental strains grew.

These results suggest that environmental isolates of *Aeromonas* sp. from sources routinely kept at low temperature (i.e., grocery store produce) are more adapted to competitive growth at low temperature. Clinical isolates may adapt to or be selected by a warmer environment. Competitive growth of *Aeromonas* sp. at 5°C is of considerable importance since refrigeration will not prevent growth, and the rapid growth of isolates at 12°C shows that only small temperature increases can lead to rapid propagation of these bacteria in produce.

Thirty (67%) and 27 (60%) of 45 *Aeromonas* isolates from grocery store produce were cytotoxic and hemolytic, respectively. The specific public health significance of these findings is unknown, since the minimum infectious dose for this microbe has not been determined. However, the presence of significant levels of cytotoxic and hemolytic *Aeromonas* sp. in many kinds of produce indicates that food may play an important role in the epidemiology of human *A. hydrophila* gastroenteritis. Unlike meat, produce is commonly eaten uncooked; therefore, chances for ingestion of significant inocula of cytotoxic *A. hydrophila* are greatly increased.

Popoff and Veron (26) reorganized the genus into a simpler taxonomy than formerly used. These changes have largely been adopted and are reflected in the most recent edition of *Bergey's Manual* (26). By this classification, the identification as *A. hydrophila* was a good indicator of cytotoxicity. Other investigators report the highest percentage of enterotoxigenic isolates from strains designated as *A. hydrophila* (7, 8).

Burke et al. (7, 8) classified VP-negative strains which did not oxidize gluconate or produce gas from glucose as nonenterotoxigenic strains. Our results correlate closely, since only 1 (6%) of 16 *A. caviae* isolates was cytotoxic. Turnbull et al. (32) reported only 11% of *A. caviae* isolates to be enterotoxigenic, while 95% of *A. hydrophila* isolates were enterotoxigenic. It seems clear that species identified as *A. hydrophila* are more likely to be cytotoxic than isolates of *A. caviae*.

Some differences between percentages of cytotoxic isolates recovered from grocery store produce may be due to the source of isolation or the method of cytotoxin detection. Burke et al. (6) showed by the suckling mouse assay that nonenterotoxigenic strains may produce cytotoxins in cell cultures. However, determination of cytotoxicity via cell culture generally correlated well with the suckling mouse assay when water isolates were tested (9).

Other investigators have correlated *Aeromonas* toxigenicity with hemolysin production (1, 3, 9, 32). For instance, Burke et al. (7, 8) reported that 97% of cytotoxic *Aeromonas* strains could be correctly classified by the hemolysin assay. Our results were slightly lower. However, all hemolytic strains were cytotoxic, and our results suggest that hemolysis is still a primary indicator of cytotoxic activity.

Strong correlations between cytotoxicity and positive VP reaction have been reported by others (8, 13, 32). In contradistinction, Janda et al. (19) found that only 42% of 12 cytotoxic *A. hydrophila* strains from pediatric patients were VP positive. However, these VP-negative strains may have been cytotoxic *A. sobria* or *A. caviae* strains according to Popoff's classification (25).

Several investigators have reported a high correlation between cytotoxic *A. hydrophila* and a positive LDC reaction (1, 13, 19). Initially, we found a low percentage of LDC-positive strains to be cytotoxic. This was probably due to the 18- to 24-h incubation of the API 20E strips, since some strains are positive only after 48 to 96 h of incubation (13). When LDC assays were repeated with broth tube cultures (GIBCO), 28 (93%) of 30 cytotoxic *A. hydrophila* strains were LDC positive, while none (0%) of the noncytotoxic isolates was. Therefore, the production of LDC is an excellent indicator of cytotoxicity when the more sensitive LDC broth is used.

The high incidence of sorbitol fermentation among cytotoxic *Aeromonas* isolates was unexpected. To our knowledge, we are the only investigators to observe this phenomenon. Our results indicate that sorbitol fermentation, while not as predictive as VP reaction, LDC production, and hemolysis, may be a helpful indicator of cytotoxic activity.

Our results establish cytotoxic *A. hydrophila* as a potential psychrotrophic food-borne pathogen ubiquitously associated with grocery store produce. In addition, all strains identified as *A. hydrophila* produced a cytotoxin, and the presence of this toxin correlated closely with hemolysis and LDC production. However, confirmation that these organisms are potential enteric pathogens is dependent on separate research with animal models or volunteer studies to definitively establish *A. hydrophila* as a gastrointestinal pathogen.

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